PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12P 21/00, C07K 14/415, A61K 38/16, 7/48 — A1

(11) International Publication Number: WO 96/20284

(43) International Publication Date: 4 July 1996 (04.07.96)

(21) International Application Number: PCT/EP95/05084

(22) International Filing Date: 21 December 1995 (21.12.95)

(30) Priority Data:
MI94A002663 28 December 1994 (28.12.94) IT

(71) Applicant *(for all designated States except US)*: INDENA S.P.A. [IT/IT]; Viale Ortles, 12, I-20139 Milano (IT).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: BOMBARDELLI, Ezio [IT/IT]; Viale Ortles, 12, I-20139 Milano (IT). PONZONE, Cesare [IT/IT]; Viale Ortles, 12, I-20139 Milano (IT).

(74) Agent: MINOJA, Fabrizio; Studio Consulenza Brevettuale, Via Rossini, 8, I-20122 Milano (IT).

(81) Designated States: AL, AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: HYDROXYPROLINE-RICH PROTEINS AND PHARMACEUTICAL AND COSMETIC FORMULATIONS CONTAINING THEM

(57) Abstract

The invention relates to hydroxyproline-rich glycoproteins, which can be obtained by acid alcohol extraction from *Taxus spp.*, *Gingko biloba*, *Lycopersicum esculentum* and *Daucus carota* cell cultures, having the following characteristics: average molecular weight 20,000 Daltons with variability interval 12,000 to 38,000, determined by means of gel permeation and electrophoresis; high solubility in water.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam

HYDROXYPROLINE-RICH PROTEINS AND PHARMACEUTICAL AND COSMETIC FORMULATIONS CONTAINING THEM

The present invention relates to hydroxyproline-rich glycoproteins which can be obtained from vegetable sources, and to the pharmaceutical and cosmetic use thereof.

More precisely, the invention relates to hydroxyproline-rich glycoproteins, which can be obtained by acid alcohol extraction from Taxus spp., Gingko biloba, Lycopersicum esculentum and Daucus carota cell cultures, having the following characteristics:

- average molecular weight 20,000 Daltons with variability interval 12,000 to 38,000, determined by means of gel permeation and electrophoresis;
- solubility in acid aqueous solutions.

Some glycoproteins of animal origin, such as collagen and proteoglycans, are known to exert a beneficial action on the skin when applied topically as such or incorporated in suitable formulations.

Collagen, which is a glycoprotein rich in proline and hydroxyproline, is especially used as such or combined with other polypeptide bases in the treatment of wrinkles and other unaesthetic blemishes linked to poor skin hydration and elasticity. The animal origin of collage, however, limits its use because of the risks of contamination from viruses and toxins. Though the compounds of vegetable origin do not involve these risks, so far their use in cosmetics has been quite limited: for examples, cosmetic formulations are known which contain raw extracts of such plants as Aloe or

even entire minced vegetables such as avocado.

Vegetable glycoproteins, called extensines, that are produced from vegetable cells in the proliferation stage and have a similar structure to animal collagen, are known. EP-A-0 533 4078 discloses the cosmetic use of extensines having an average molecular weight above 100,000 Daltons. However, the methods for the extraction of extensines described to date, which involve the extraction of vegetable materials of various origin by means of aqueous saline solutions, followed by purification with strong acids such as trichloroacetic acid, do not allow to obtain suitable products for cosmetics, due to problems concerning solubility, stability, repeatability and consistency of their chemical-physical characteristics.

It has now been found that it is possible to obtain hydroxyproline-rich glycoproteins, structurally similar to the above described extensines but with a lower molecular weight and a higher solubility in acid aqueous solutions, by means of a procedure comprising the in vitro culture of cells of selected plants and the extraction, with acid alcoholic solutions, of the cells grown in a suitable medium.

The glycoproteins obtainable according to the invention have hydrating, film-forming, toning and cicatrizant properties higher than those of collagen. The glycoproteins of this invention can therefore be employed in cosmetic or dermatologic formulations for the treatment of dry skin, psoriasis, ichtyosis, dandruff, keratosis, wrinkles, acne, eczema, inflammatory dermatosis, ageing of the skin and all the

other applications for which the use of animal collagen has been proposed.

The aqueous solutions of the glycoproteins of the invention remain stable without any polymerisation of the glycoproteins leading to the formation of insoluble products. In addition, the viscosity of these solutions is particularly high and not dependent on the concentrations; 0.1% concentrations surprisingly have the same film-forming and hydrating power equal as 1% collagen or 5% vegetable albumin solutions.

The vegetable material to be extracted is obtained from fermenter cultures of Taxus spp., Gingko biloba, Lycopersicum esculentum and Daucus carota cells. The use of cells from the species Taxus spp., Gingko biloba and Lycopersicum esculentum is particularly preferred. The cell culture techniques are conventional and include the suspension culture starting from callus cultures from various parts of the plants such as leaves, bark, roots, trunk or seeds, as described by Dobbs and Roberts, Experiments in Plant Tissue Culture, 2nd ed. Cambridge University Press, New York, 1985.

The vegetable tissue of the callus, following sterilisation and optional addition of antibacterials, is typically used for the inoculum of suitable liquid culture media as described in the above mentioned Manual by Dobbs and Roberts. A particularly suitable medium for this invention is the Murashige and Skoog medium. The addition of specific additives such as proline, reducing agents, ethylene or compounds capable of releasing ethylene such as Ethephon or L-aminocyclo-propanecarboxylic acid, may be suitable to increase

productivity in the desired glycoproteins.

The use of naphthylacetic acid as the as auxin, 6-(γ,γ-dimethylamino)-purine as the cytokinin, vitamins and 3% saccharose as the carbon source is preferred. The addition of vitamin C may be suitable, depending on the material chosen, to prevent the final product from browning.

The fermentation time may vary from 3 to 12 days and is preferably between 5 and 6 days. Once the fermentation has been completed, the culture medium is centrifuged and the cellular mass is extracted by means of alcohols, preferably ethanol, in the presence of diluted mineral acids, preferably hydrochloric or sulphuric acid. This procedure inactivates some enzymes that may jeopardise the stability of the glycoproteins of the invention, specifically of polyphenoloxidase and tyrosine oxidase which favour the polymerization of glycoproteins with the consequent formation of insoluble products.

The alcohol extraction in the presence of mineral acids allows the complete extraction of basic glycoproteins and has proved to be extremely selective to this end. Other water-mixable alcohols, such as methanol or isopropanol, can be used besides ethanol. The resulting hydroalcoholic extracts are neutralised and then concentrated and heated to a temperature of 70°C to 100°C, preferably around 80°C, up to complete precipitation of the denatured proteins. The suspension is then clarified by concentration and the fluid is subjected to fractional ultrafiltration to remove high and low molecular weight substances. Ultrafiltration is

performed by means of polysulphonic membranes having cut-off of 10,000 Daltons to 40,000 Daltons, such as Centricon^{R} or Romicon^{R}, whose fibres may be hollow or, alternatively, coiled. The resulting filtered product is electrodialysed to remove undesired substances such as salts and low molecular weight sugars. After filtration and dialysis, the resulting solution can be used as such in cosmetic or pharmaceutical preparations or it can be concentrated to a lower volume and then lyophilised or atomised.

The analytical characterization of the products of the invention was carried out by gel permeation using a high-pressure liquid chromatograph consisting of a Waters pump unit and provided with a Ultrahydrogel Linear Waters^{R} column battery 30 cm x 0.5 cm and Waters UV absorption detector, model 484. An aqueous solution containing 0.067 M monopotassium phosphate, 0.1 M NaCl and 6 x 10^{-4} M NaN_3 was used as the eluent. The glycoprotein samples to be analysed are dissolved in the same eluent solution (3 mg/10 ml) and scalar amounts of the substance as well as the reference substances selected as molecular weights between Cytochrome C (12,400 Daltons) and dextran blue (2,000,000 Daltons); alternatively or simultaneously the products or their intermediates can be determined by electrophoresis on 12.5% polyacrylamide gel and 4% stacking gel. The samples to be analysed are dissolved in a buffer containing SDS and 0.1% mercaptoethanol while depositing quantities between 100 mg and 300 mg. The migration is carried out at a constant current at 20 mA for 4 hours. A gauging curve is drawn with 5 standard weights (7kD,

14kD, 24kD, 54kD and 66kD). Weights of 22.5kD and 25kD are calculated from this gauging curve for the two main bands and weights of 31kD and 34kD are calculated for the less intense bands. The procedures described here allow mixtures of products with comparable molecular weights and comparable amino acid compositions to be obtained from the various cell explants starting from different plants. The results of the amino acid analysis of glycoproteins extracted from Ginkgo biloba cells are shown below as an example.

Amino acid	Peak area %
Asp	4.399
Glu	4.328
Hyp	17.505
Ser	7.065
Gly	6.056
Hys	1.782
Arg	2.471
Thr	4.739
Pro	10.036
Ala	8.2
Tyr	2.388
Val	6.162
Met	1.154
Ile	2.479
Leu	5.525
Phe	1.862
Lys	14.254

The above data refer to the percentage of the total amount of amino acids present in the glycoprotein mixture. The sugars in the mixture are arabinose and

galactose. The ratio of amino acids to sugars is on average 2:1 for the various products.

As mentioned above, the products according to the invention can be used both in the pharmaceutical and cosmetic fields. For the pharmaceutical field, the product may be incorporated in gels or ointments or applied on medicated gauzes for specific treatment of burns or wounds. In this case the product is usually subjected to sterilisation or sterile filtrations and lyophilised.

The cosmetic and dermatologic preparations of the invention can be prepared according to traditional methods. Examples of administration forms include aqueous sprays, lotions, solutions, emulsions, gels, ointments and creams.

The cosmetic and dermatologic preparations of the inventions can contain hydroxyproline-rich glycoproteins in weight percentages of about 0.01% to about 50%, preferably from 0.05% to 5%, as well as conventional excipients. Given the high stability of the glycoproteins of this invention, pharmaceutical and cosmetic preparations containing above 50% of soluble hydroxyproline-rich glycoproteins can be obtained.

The glycoproteins of the invention can be added to pharmaceutical and cosmetic preparations as such or microencapsulated so as to provide a long-term hydrating action. The microcapsules can be either hydrophilic or lipophilic. The preparations of the invention may include other active principles having complementary or useful activity for the desired aims.

The invention is further illustrated by the

following examples.

Example 1

Preparation of the callus and liquid culture of Ginkgo biloba for the production of glycoproteins

An explant of young leaves of Ginkgo biloba is prepared by washing the leaves in a 0.1% Tween 80(R) solution. The laminae are sectioned in fractions of about 0.5 cm and pre-sterilised for 1 minute with 75% ethanol. The sterilisation is then completed with a 2% sodium hypochlorite solution and triple washing of the explant in sterile water. The resulting explants are transferred to a Petri dish in Murashige & Skoog medium containing 3% saccharose with the addition of Lynsmeyer & Skoog vitamins and hormones such as 2,4-dichlorophenoxyacetic acid and naphthylacetic acid. The products are incubated in the dark at 23°C for 20 days. At the end of this period, friable calli are obtained which grow easily and are moved in continuous rows by means of subcultures in the same conditions, as they can be used for propagation in a liquid medium. These calli are used to inoculate Erlenmeyer flasks containing 200 ml of Murashige & Skoog medium, with the addition of naphthylacetic acid and 6 (γ,γ-dimethylamino)-purine, Lynsmeyer & Skoog vitamins and 3% saccharose as a source of carbon. The flasks are incubated with stirring in continuous light for 4 days, after which the cell biomass is harvested for the extraction of glycoproteins.

Example 2

Preparation of glycoproteins from Ginkgo biloba cells

5 liters of the culture obtained according to

Example 1 are low-speed centrifuged and the harvested cells (1.5 kg of fresh weight) are extracted with 1.5 l of 70% ethanol containing 1% sulphuric acid. The extraction is repeated twice thereby quantitatively recovering the basic glycoproteins. After neutralisation, the extracts are filtered to remove any turbidity and concentrated under vacuum at 50°C until ethanol is completely removed. The aqueous concentrate is heated at 85°C for 30 minutes and centrifuged again to remove the precipitate, which is discarded. The resulting clear solution is ultrafiltered by means of a Centricon^{R} membrane with cut-off 40,000 Dalton limit to exclude the higher molecular weights.

The filtered product is then ultrafiltered using a hollow-fibre membrane with cut-off 10,000 Dalton to remove non-glycoprotein, low-molecular weight substances. The filtrate is then subjected to dialysis and concentrated to 1% of solid residue. 1.5 litres of a slightly viscous product is obtained, which may be used as such in cosmetic formulations. At the electrophoresis analysis, the product contained 6 bands, 4 of which had molecular weights of 16,000, 22,000, 33,000 and 36,000 Daltons.

Example 3

Preparation of glycoproteins from Lycopersicum esculentum

Following the procedure of Example 1, a cell mass from sterile buds of Lycopersicum esculentum is prepared in a 14-liter fermenter containing 10 litres of Murashige & Skoog medium added with naphthylacetic acid and 6 (γ,γ-dimethylamino)-purine, Lynsmeyer & Skoog

vitamins and 3% saccharose as a carbon source. The fermentation is carried on for 5 days at 23°C while stirring at 150 rpm in the presence of yeast extract at 0.05% concentration and with an approximately 70% concentration of dissolved oxygen. At the end of the fermentation the broth is gathered and micro-filtered through a 0.2 µm ceramic membrane to concentrate the cells. Some isopropanol containing 0.5% hydrochloric acid is added to the cell paste thus obtained and the method described in Example 2 is applied to the extracts. 3.5 litres of a solution are obtained with 0.5% dry residue. The analysis of the lyophilised solutions gave a content of 10% proline and 31% hydroxyproline, respectively.

Example 4

Cosmetic formulation

100 g of O/W emulsion contain:

SOLUTION OF THE EXAMPLE 2 OR 3	10.0 g
Acetylated lanolin alcohol PEG-10	2.0 g
Cetyl-stearyl alcohol	1.5 g
Cetyl palmitate	2.0 g
Stearic acid	7.0 g
Octyl octanoate	7.5 g
Potassium cetyl phosphate	0.5 g
Preservatives	q.s.
Fragrance	q.s.
Purified water	q.s. to 100 g

Example 5

Cosmetic formulation

100 g of O/W emulsion contain:

SOLUTION OF THE EXAMPLE 2 OR 3	10.0 g

Cetyl stearyl glucoside	5.0 g
Jojoba oil	10.0 g
Isopropyl myristate	8.0 g
Dimethicone	0.5 g
Antioxidant	q.s.
Preservatives	q.s.
Fragrance	q.s.
Purified water	q.s. to 100 g

CLAIMS

1. Hydroxyproline-rich glycoproteins, obtainable by acid-alcohol extraction from Taxus spp., Gingko biloba, Lycopersicum esculentum and Daucus carota cell cultures, having the following characteristics:

- average molecular weight 20,000 Daltons with variability interval 12,000 to 38,000, determined by means of gel permeation and electrophoresis;
- high solubility in water.

2. A process for the preparation of the glycoproteins of Claim 1, which process comprises:

a) culture of Taxus spp., Gingko biloba, Lycopersicum esculentum and Daucus carota cells in a liquid medium for a time from 3 to 12 days;

b) extraction of the cell mass from with water-mixable alcohols in the presence of diluted mineral acids;

c) neutralisation, concentration and heating of the extracts at temperatures between 70°C and 100°C;

d) centrifugation, fractional ultrafiltration and dialysis.

3. Cosmetic and pharmaceutical preparations containing the glycoproteins of Claim 1 as active principle, having hydrating, film-forming, toning and cicatrizant properties.

4. Preparations according to Claim 3 in the form of aqueous sprays, lotions, solutions, emulsions, gels, ointments, creams, and medicated gauzes.

A. CLASSIFICATION OF SUBJECT MATTE
IPC 6 C12P21/00 C07K[illegible]15 A61K38/16 A61K7/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 533 408 (REVLON, INC.) 24 March 1993 cited in the application see page 3, line 3 - page 5, line 3	1-4
A	PLANT PHYSIOL., vol. 84, 1987, pages 820-825, XP000569867 JOEL P. STAFSTROM ET AL.: "A SECOND EXTENSIN-LIKE HYDROXYPROLINE-RICH GLYCOPROTEIN FROM CARROT CELL WALLS."	

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
3 May 1996	10. 05. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Rempp, G

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-533408	24-03-93	JP-A- 6107517	19-04-94
		US-A- 5443855	22-08-95
		ZA-A- 9207075	16-03-94